

EXHIBIT C

Hypertension



Learn and Live

Na⁺/Ca²⁺ Exchanger: Target for Oxidative Stress in Salt-Sensitive Hypertension

M. Tino Unlap, Elizabeth Bates, Corey Williams, Peter Komlosi, Iantha Williams,
Gergely Kovacs, Brian Siroky and P. Darwin Bell

Hypertension 2003;42;363-368; originally published online Jul 28, 2003;

DOI: 10.1161/01.HYP.0000084060.54314.F5

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX
72514

Copyright © 2003 American Heart Association. All rights reserved. Print ISSN: 0194-911X. Online
ISSN: 1524-4563

The online version of this article, along with updated information and services, is
located on the World Wide Web at:

<http://hyper.ahajournals.org/cgi/content/full/42/3/363>

Subscriptions: Information about subscribing to Hypertension is online at
<http://hyper.ahajournals.org/subscriptions/>

Permissions: Permissions & Rights Desk, Lippincott Williams & Wilkins, a division of Wolters
Kluwer Health, 351 West Camden Street, Baltimore, MD 21202-2436. Phone: 410-528-4050. Fax:
410-528-8550. E-mail:
journalpermissions@lww.com

Reprints: Information about reprints can be found online at
<http://www.lww.com/reprints>

Na⁺/Ca²⁺ Exchanger

Target for Oxidative Stress in Salt-Sensitive Hypertension

M. Tino Unlap, Elizabeth Bates, Corey Williams, Peter Komlosi, Iantha Williams,
Gergely Kovacs, Brian Siroky, P. Darwin Bell

Abstract—The Na⁺/Ca²⁺ exchanger regulates intracellular calcium ([Ca²⁺]_i), and attenuation of Na⁺/Ca²⁺ exchange by oxidative stress might lead to dysregulation of [Ca²⁺]_i. We have shown that the Na⁺/Ca²⁺ exchanger differs functionally and at the amino acid level between salt-sensitive and salt-resistant rats. Therefore, the purpose of these studies was to determine how oxidative stress affects the activities of the 2 Na⁺/Ca²⁺ exchangers that we cloned from mesangial cells of salt-resistant (RNCX) and salt-sensitive (SNCX) Dahl/Rapp rats. The effects of oxidative stress on exchanger activity were examined in cells expressing RNCX or SNCX by assessing ⁴⁵Ca²⁺ uptake (reverse mode) and [Ca²⁺]_i elevation (forward mode) in the presence and absence of H₂O₂ and peroxynitrite. Our results showed that ⁴⁵Ca²⁺ uptake in SNCX cells was attenuated at 500 and 750 μmol/L H₂O₂ (63±12% and 25±7%, respectively; n=16) and at 50 and 100 μmol/L peroxynitrite (47±9% and 22±9%, respectively; n=16). In RNCX cells, ⁴⁵Ca²⁺ uptake was attenuated at only 750 and 100 μmol/L H₂O₂ and peroxynitrite (61±9% and 63±6%, respectively; n=16). In addition, the elevation in [Ca²⁺]_i was greater in SNCX cells than in RNCX cells in response to 750 μmol/L H₂O₂ (58±5.5 vs 17±4.1 nmol/L; n=13) and 100 μmol/L peroxynitrite (33±5 vs 11±6 nmol/L; n=19). The enhanced impairment of SNCX activity by oxidative stress might contribute to the dysregulation of [Ca²⁺]_i that is found in this model of salt-sensitive hypertension. (*Hypertension*. 2003;42:363-368.)

Key Words: ion transport ■ oxidative stress ■ hypertension, sodium-dependent ■ calcium

The Na⁺/Ca²⁺ exchanger (NCX) is an important regulator of intracellular calcium concentration ([Ca²⁺]_i) in a number of different cell types, including afferent arteriole vascular smooth muscle cells and mesangial cells.¹⁻⁵ Utilizing the electrochemical gradient for Na⁺, the exchanger transports 1 Ca²⁺ ion for every 3 Na⁺ ions that enter the cell. Thus, the exchanger regulates the level of [Ca²⁺]_i by serving as a major Ca²⁺ efflux pathway.

In our previous studies, we identified Na⁺/Ca²⁺ exchange activity in afferent arterioles and mesangial cells of salt-resistant and salt-sensitive Dahl/Rapp rats.^{2,4,5} Although exchanger activity was present in these contractile cells from both strains of rat, there were differences in exchanger regulation.^{2,4,5} Specifically, NCX activity was upregulated by protein kinase C in afferent arterioles and mesangial cells of salt-resistant but not salt-sensitive rats.^{2,4} To examine the basis of this differential regulation by protein kinase C, we cloned and sequenced the NCX from mesangial cells of salt-resistant (RNCX) and salt-sensitive (SNCX) Dahl/Rapp rats.⁵ We found that RNCX and SNCX, both isoforms of NCX1, are 100% homologous in the C-terminus membrane-spanning domains but differ by 1 amino acid (amino acid 218) in the N-terminus membrane-spanning domains. This

amino acid residue is isoleucine in RNCX but phenylalanine in SNCX. The 2 isoforms also differ at the alternative splice site, where SNCX is encoded by exons B, D, and F and RNCX is encoded by exons B and D. Functional studies in opossum kidney proximal tubule (OK-PTH) cells expressing either RNCX or SNCX confirmed that RNCX but not SNCX was activated by protein kinase C.⁵ In addition, recent studies demonstrated that SNCX has an impaired ability to regulate agonist-induced [Ca²⁺]_i increases.^{5,6}

Dysregulation of [Ca²⁺]_i can lead or contribute to deleterious pathogenic conditions, including neurodegenerative diseases⁷ and salt-sensitive hypertension.^{1,8} Recently, there has also been a preponderance of evidence implicating oxidative stress in the pathogenesis of hypertension,^{9,10} and this effect of oxidative stress might occur, at least in part, through alterations in [Ca²⁺]_i dynamics. Because the NCX is 1 of the primary regulators of [Ca²⁺]_i, alterations in [Ca²⁺]_i dynamics under oxidative stress conditions might be attributed, at least to some extent, to dysregulation of the NCX. Susceptibility of the exchanger to oxidative stress was previously found in synaptic plasma membranes of Chinese hamster ovary-K1 cells expressing NCX, in which treatment with AAPH (a peroxy-radical generator) or peroxynitrite attenuated ex-

Received March 31, 2003; first decision April 18, 2003; revision accepted June 17, 2003.

From the Nephrology Research and Training Center, Departments of Medicine and Physiology, Division of Nephrology, University of Alabama at Birmingham.

Correspondence to M. Tino Unlap, PhD, University of Alabama at Birmingham, UAB Station, 865 Sparks Ctr, Birmingham, AL 35294. E-mail unlap@uab.edu

© 2003 American Heart Association, Inc.

Hypertension is available at <http://www.hypertensionaha.org>

DOI: 10.1161/01.HYP.0000084060.54314.F5

changer activity through the formation of high-molecular-weight aggregates and NCX fragmentation.¹¹ In addition, Kaplan et al¹² found that induction of oxidative stress in gerbil forebrain synaptosomes by treatment with Fe-EDTA (200 $\mu\text{mol/L}$, 30 minutes) resulted in a 47% reduction in exchange activity. Thus, the NCX appears to be sensitive to oxidative stress. This led us to the present studies in which we tested the idea that differences between RNCX and SNCX at the amino acid level might result in differential sensitivities of these 2 isoforms to oxidative stress.

Methods

Cell Culture

OK-PTH cells (American Type Culture Collection, Manassas, Va) expressing either RNCX or SNCX NCX isoforms were grown and maintained as previously described.^{2,4,5}

Reverse-Mode Exchanger Activity Measurements

Although the physiologic function of the exchanger is to extrude Ca^{2+} in exchange for Na^+ , the reverse mode of the exchanger can be assessed by using a Na^+ -dependent $^{45}\text{Ca}^{2+}$ -uptake assay.^{2,4,5} The effects of oxidative stress on exchanger activity were assessed by performing $^{45}\text{Ca}^{2+}$ uptake in the presence of H_2O_2 (500 or 750 $\mu\text{mol/L}$) or peroxynitrite (50 or 100 $\mu\text{mol/L}$). Exchange activity is presented as $\text{nmol } ^{45}\text{Ca}^{2+}/\text{mg protein per minute}$.

Forward-Mode Exchanger Activity Measurements

OK-PTH cells expressing either RNCX (ROK) or SNCX (SOK) were grown on coverslips to $\approx 80\%$ confluence. To load the cells with dye, coverslips were incubated in serum-free medium containing 17 $\mu\text{mol/L}$ fura 2-AM (TEF Labs) dissolved in dimethyl sulfoxide for 1 hour at 37°C . The medium was removed, and the coverslips were placed in 150-Ringer's solution (150 mmol/L NaCl , 5 mmol/L KCl , 1 mmol/L MgSO_4 , 1.6 mmol/L Na_2HPO_4 , 0.4 mmol/L NaH_2PO_4 , 5 mmol/L D-glucose, 1.5 mmol/L CaCl_2 , and 10 mmol/L HEPES). $[\text{Ca}^{2+}]_i$ measurements were then performed by using dual-excitation-wavelength fluorescence spectroscopy (Photon Technology International). Excitation wavelengths were set at 340 and 380 nm, and emission wavelength was set at 510 nm. Data points were collected, with Photon Technology International software, at a rate of 5 points per second. Baseline fura-2 ratios were measured in cells bathed in 150-Ringer's solution for at least 100 seconds, and experiments were then performed only when the ratio remained stable. The effects of oxidative stress were assessed by perfusion with Ringer's solution containing either 750 $\mu\text{mol/L}$ H_2O_2 or 100 $\mu\text{mol/L}$ peroxynitrite, and the fura-2 ratio was monitored continuously for at least 1000 seconds. All solutions had a pH of 7.4, and temperature was maintained at 37°C .

Calibration of $[\text{Ca}^{2+}]_i$

Calibrations were performed to convert fura-2 ratios to $[\text{Ca}^{2+}]_i$ values. $[\text{Ca}^{2+}]_i$ was calculated by the equation described by Grynkiewicz et al,¹³ and the values for the variables in this equation were obtained in ROK or SOK cells, as previously described.^{5,6} $\Delta[\text{Ca}^{2+}]_i$ is the difference between $[\text{Ca}^{2+}]_i$ in the absence (baseline) and presence of treatment. The rate is calculated by taking the initial slope at which calcium begins to increase and is expressed as $\text{nmol } \text{Ca}^{2+}/\text{s}$.

Immunoblotting With NCX-Specific Antibody

The effect of oxidative stress on NCX levels was determined by treating cells expressing RNCX or SNCX with a peroxy-radical generator, 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH, 5 mg/mL), or H_2O_2 (750 $\mu\text{mol/L}$) for 30 minutes. Total protein was isolated and assessed (50 μg) by immunoblotting with a rabbit polyclonal NCX antibody (Alpha Diagnostic International), as previously described.^{5,6} Before immunodetection, the polyvinylidene

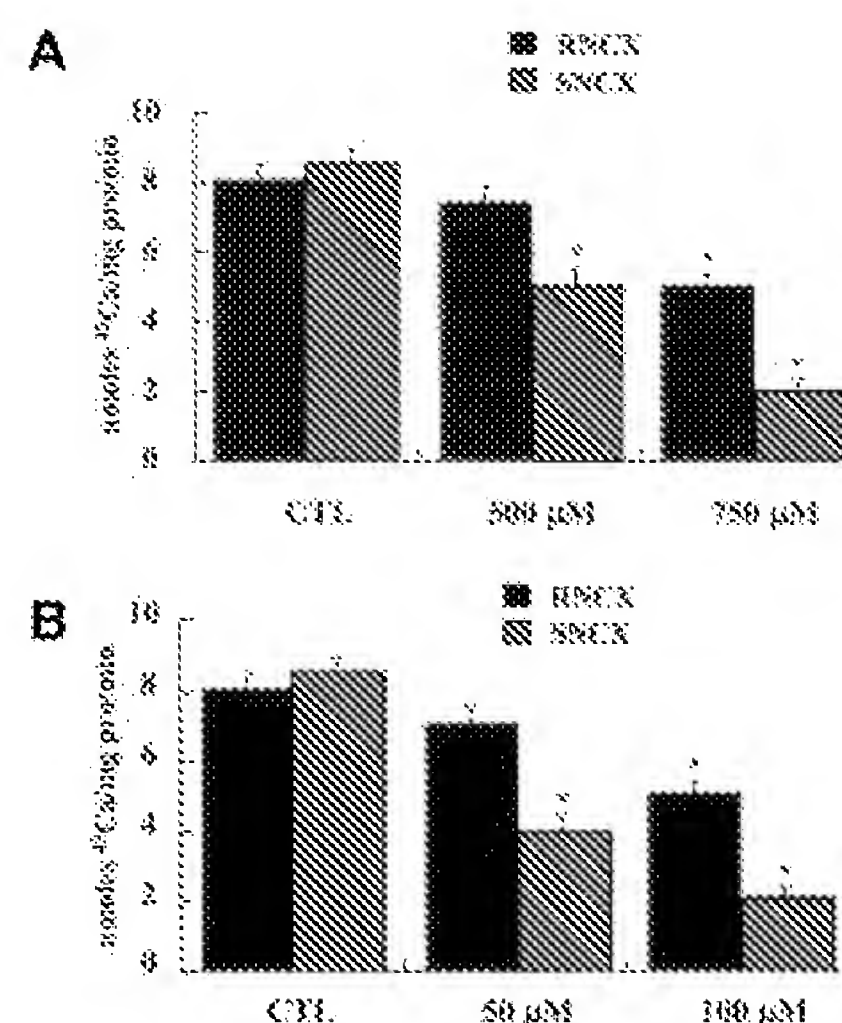


Figure 1. SNCX demonstrates greater sensitivity to H_2O_2 and ONOO^- than RNCX. Effects of oxidative stress on reverse-mode $\text{Na}^+/\text{Ca}^{2+}$ exchange activity was assessed by performing $^{45}\text{Ca}^{2+}$ uptake assays in the presence of (A) H_2O_2 (500 or 750 $\mu\text{mol/L}$) or (B) peroxynitrite (50 or 100 $\mu\text{mol/L}$) in OK-PTH cells expressing RNCX or SNCX. Exchange activity is presented as $\text{nmol } ^{45}\text{Ca}^{2+}/\text{mg protein per minute}$ for cells in the presence of a Na^+ gradient minus that for cells in the absence of a Na^+ gradient. $\text{Na}^+/\text{Ca}^{2+}$ exchange activities were compared between control (CTL) and treated samples by ANOVA. Data are mean \pm SEM. * $P < 0.05$ vs control; $n = 16$.

difluoride membrane was stained with 0.1% Ponceau S (Sigma) for 3 minutes to evaluate the loading of proteins in each lane and then rinsed in double-distilled water. Only equally loaded lanes were used for comparison to assess the effect of each treatment.

Cell Viability Assay

To assess the viability of OK-PTH cells expressing RNCX or SNCX in the presence of AAPH (5 mg/mL) or H_2O_2 (750 $\mu\text{mol/L}$), cells were grown to confluence on coverslips and either left untreated or treated with AAPH or H_2O_2 for 30 minutes, washed, and perfused with 150-Ringer's solution in the presence of the fluorescence dye 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester (BCECF, AM). The ability of cells to take up BCECF, to hydrolyze the ester, and to retain the fluorophore intracellularly is routinely used to assess cell viability.¹⁴ BCECF fluorescence was measured by dual-excitation-wavelength fluorescence spectroscopy (Photon Technology International). Excitation wavelengths were set at 440 and 500 nm, and emission wavelength was set at 530 nm. Data points were collected, with Photon Technology International software, at a rate of 5 points per second and expressed as counts per second.

Statistics

Data analysis was carried out by ANOVA and an unpaired t test. The results are presented as mean \pm SEM. A probability value < 0.05 was considered statistically significant.

Results

Effects of Oxidative Stress on Reverse-Mode $\text{Na}^+/\text{Ca}^{2+}$ Exchange Activity

To directly assess the effect of oxidative stress on exchanger activity, $\text{Na}^+/\text{Ca}^{2+}$ exchange was examined in OK-PTH cells expressing RNCX or SNCX by measuring Na^+ -dependent $^{45}\text{Ca}^{2+}$ uptake in the presence or absence of H_2O_2 (500 or 750 $\mu\text{mol/L}$) or peroxynitrite (50 or 100 $\mu\text{mol/L}$). Cells expressing SNCX were more sensitive to H_2O_2 (Figure 1A) and

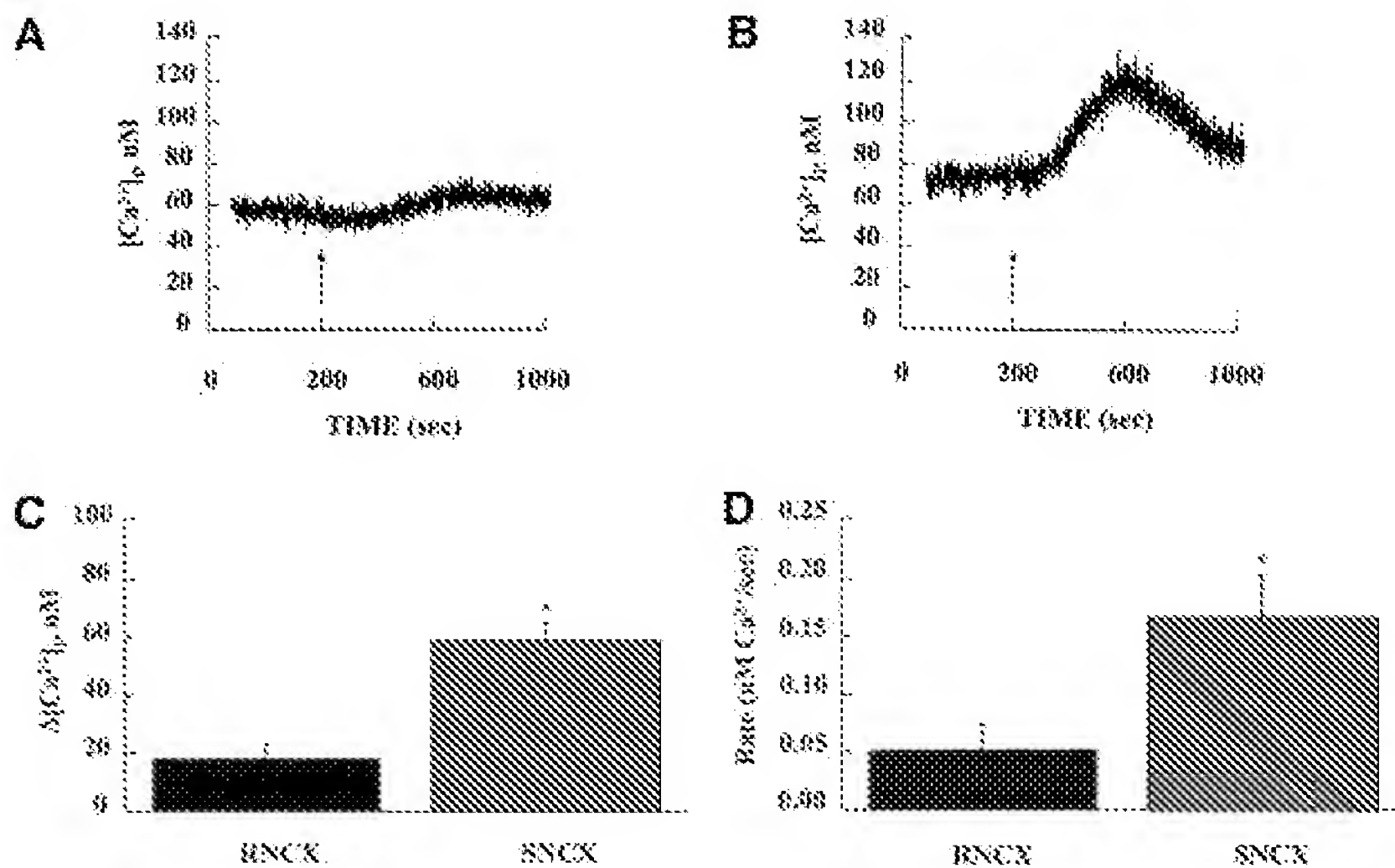


Figure 2. H_2O_2 elevates $[\text{Ca}^{2+}]_i$ to a greater degree in cells expressing SNCX than in cells expressing RNCX. Effects of H_2O_2 on $[\text{Ca}^{2+}]_i$ were assessed by perfusing fura-2-loaded cells expressing RNCX or SNCX with H_2O_2 (750 $\mu\text{mol/L}$). Representative calcium tracings are shown for effects of H_2O_2 on $[\text{Ca}^{2+}]_i$ in cells expressing RNCX (A) or SNCX (B), change in $[\text{Ca}^{2+}]_i$ ($\Delta[\text{Ca}^{2+}]_i$) (C), and the rate of increase in $[\text{Ca}^{2+}]_i$ in response to H_2O_2 (D). Effects of H_2O_2 on $\Delta[\text{Ca}^{2+}]_i$ and the rate of increase in $[\text{Ca}^{2+}]_i$ were compared between cells expressing RNCX and SNCX by ANOVA. Data are mean \pm SEM. * $P < 0.05$ vs RNCX; $n = 13$.

peroxynitrite (Figure 1B) than cells expressing RNCX. H_2O_2 , at 500 $\mu\text{mol/L}$, attenuated exchange activity in cells expressing SNCX ($63 \pm 12\%$, $n = 16$) though not significantly affecting the activity of RNCX ($93 \pm 10\%$, $n = 16$). At 750 $\mu\text{mol/L}$, H_2O_2 attenuated the activities of both RNCX ($61 \pm 9\%$, $n = 16$) and SNCX ($25 \pm 7\%$, $n = 16$). Peroxynitrite, at 50 $\mu\text{mol/L}$, attenuated the activity of SNCX ($47 \pm 9\%$, $n = 16$) but not RNCX ($94 \pm 12\%$, $n = 16$). At 100 $\mu\text{mol/L}$, peroxynitrite attenuated the activities of both RNCX ($63 \pm 6\%$, $n = 16$) and SNCX ($22 \pm 9\%$, $n = 16$).

Effects of Oxidative Stress on $[\text{Ca}^{2+}]_i$ Regulation in Cells Expressing RNCX or SNCX

The effects of H_2O_2 and peroxynitrite on $[\text{Ca}^{2+}]_i$ in OK-PTH cells expressing the 2 renal exchanger isoforms were also tested. This was accomplished by examining the change in $[\text{Ca}^{2+}]_i$ in response to H_2O_2 and peroxynitrite. Cells expressing RNCX or SNCX both showed increases in $[\text{Ca}^{2+}]_i$ in the presence of H_2O_2 (Figures 2A and 2B) or peroxynitrite (Figures 3A and 3B). However, the increase in $[\text{Ca}^{2+}]_i$ was much greater for cells expressing SNCX compared with RNCX-expressing cells in response to H_2O_2 (58 ± 5.5 vs 17 ± 4.1 nmol/L, $n = 13$) or peroxynitrite (33 ± 5 vs 11 ± 6

nmol/L, $n = 19$), (Figures 2C and 3C, respectively). The significant difference in $[\text{Ca}^{2+}]_i$ responsiveness to oxidative stress was due to the rapid rate of increase in $[\text{Ca}^{2+}]_i$ in cells expressing SNCX, which was 3.4- (Figure 2D) and 4.8- (Figure 3D) fold greater than that for cells expressing RNCX in response to H_2O_2 and peroxynitrite, respectively.

SNCX and RNCX Exchanger Levels

We previously reported in earlier studies that OK-PTH cells did not functionally express the exchanger, as evaluated by Na^+ -dependent ^{45}Ca uptake,⁵ and that 2 OK-PTH cell lines stably transfected with RNCX or SNCX express comparable levels of exchanger proteins.⁶ To test whether oxidative stress attenuates $\text{Na}^+/\text{Ca}^{2+}$ exchange activity by reducing exchanger protein levels, immunoblot analysis was carried out on exchanger protein expression in response to AAPH (5 mg/mL) and H_2O_2 (750 $\mu\text{mol/L}$) treatment, and the results (Figure 4) demonstrated that oxidative stress attenuates NCX protein levels.

Cell Viability Assay

The effect of treatments on cell viability was assessed by examining the rate at which each cell line took up BCECF in

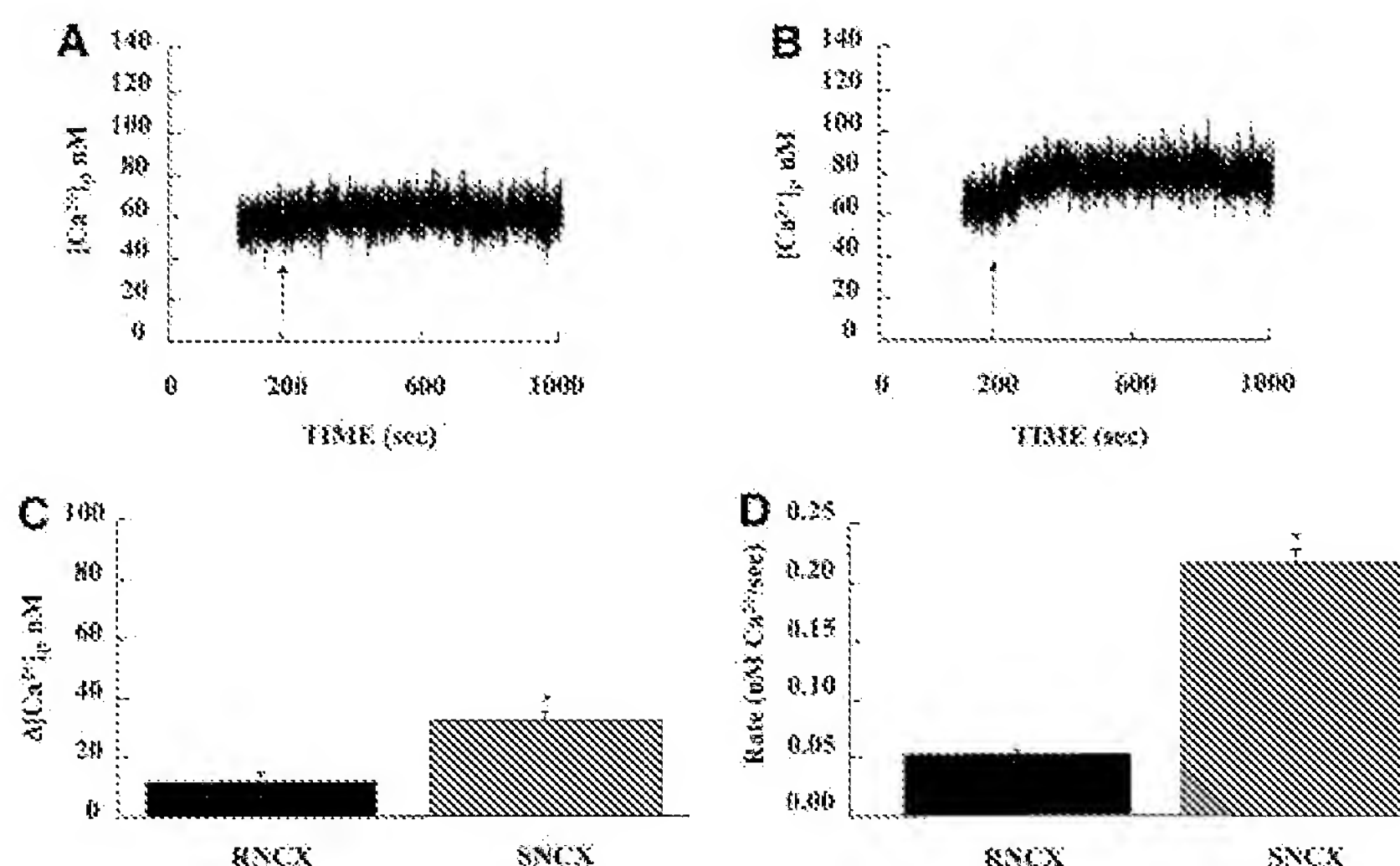


Figure 3. Peroxynitrite elevates $[\text{Ca}^{2+}]_i$ to a greater degree in cells expressing SNCX than in cells expressing RNCX. Effects of peroxynitrite on $[\text{Ca}^{2+}]_i$ were assessed by perfusing fura-2-loaded cells expressing RNCX or SNCX with peroxynitrite (250 $\mu\text{mol/L}$). Representative calcium tracings show effects of peroxynitrite on $[\text{Ca}^{2+}]_i$ in cells expressing RNCX (A) or SNCX (B), change in $[\text{Ca}^{2+}]_i$ ($\Delta[\text{Ca}^{2+}]_i$) (C), and the rate of increase in $[\text{Ca}^{2+}]_i$ in response to peroxynitrite (D). Effects of peroxynitrite on $\Delta[\text{Ca}^{2+}]_i$ and the rate of increase in $[\text{Ca}^{2+}]_i$ were compared between cells expressing RNCX and SNCX by ANOVA. Data are mean \pm SEM. * $P < 0.05$ vs RNCX; $n = 19$.

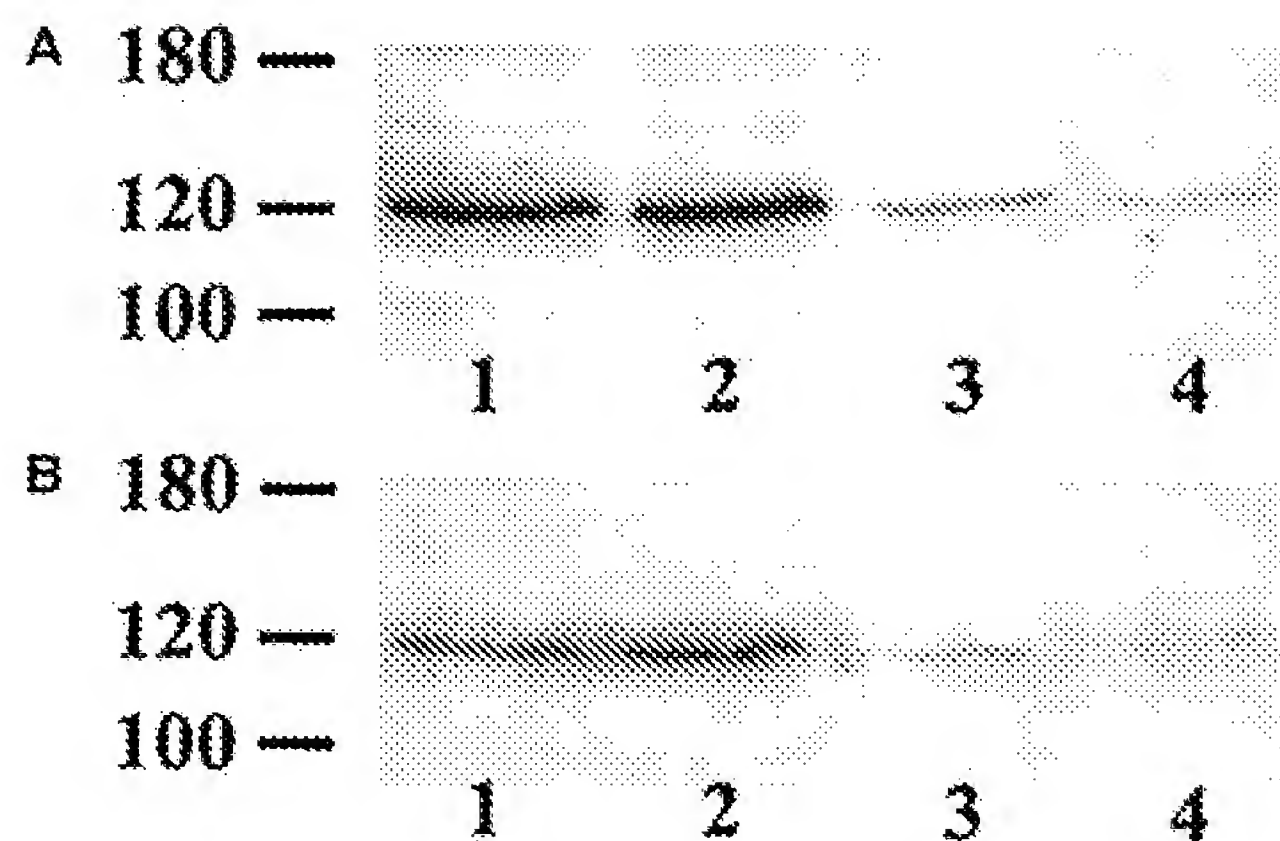


Figure 4. AAPH and H_2O_2 attenuate NCX levels. Cell lysates from OK-PTH cells expressing RNCX or SNCX, which were either left untreated or treated with AAPH (5 mg/mL) or H_2O_2 , were isolated as described in Methods. Samples were fractionated on 7.5% sodium dodecyl sulfate polyacrylamide gels, transferred to polyvinylidene difluoride membranes, and immunodetected with a rabbit polyclonal NCX antibody. Representative immunoblots show the 120-kDa band in AAPH- (A) and H_2O_2 - (B) treated cell extracts from untreated (1, 2) or treated (3, 4) cells expressing RNCX (1, 3) or SNCX (2, 4).

the absence and presence of AAPH (5 mg/mL) or H_2O_2 (750 $\mu\text{mol/L}$). The results (Figure 5) showed that neither AAPH nor H_2O_2 significantly affected the viability of OK-PTH cells expressing RNCX or SNCX.

Discussion

Oxidative stress, marked by excessive levels of reactive oxygen species, has been implicated in the pathophysiology of hypertension in 2 rat models, the spontaneously hypertensive rat and the salt-sensitive Dahl/Rapp rat. In the spontaneously hypertensive rat, reducing oxidative stress by treatment with tempol, a superoxide dismutase mimetic, or by

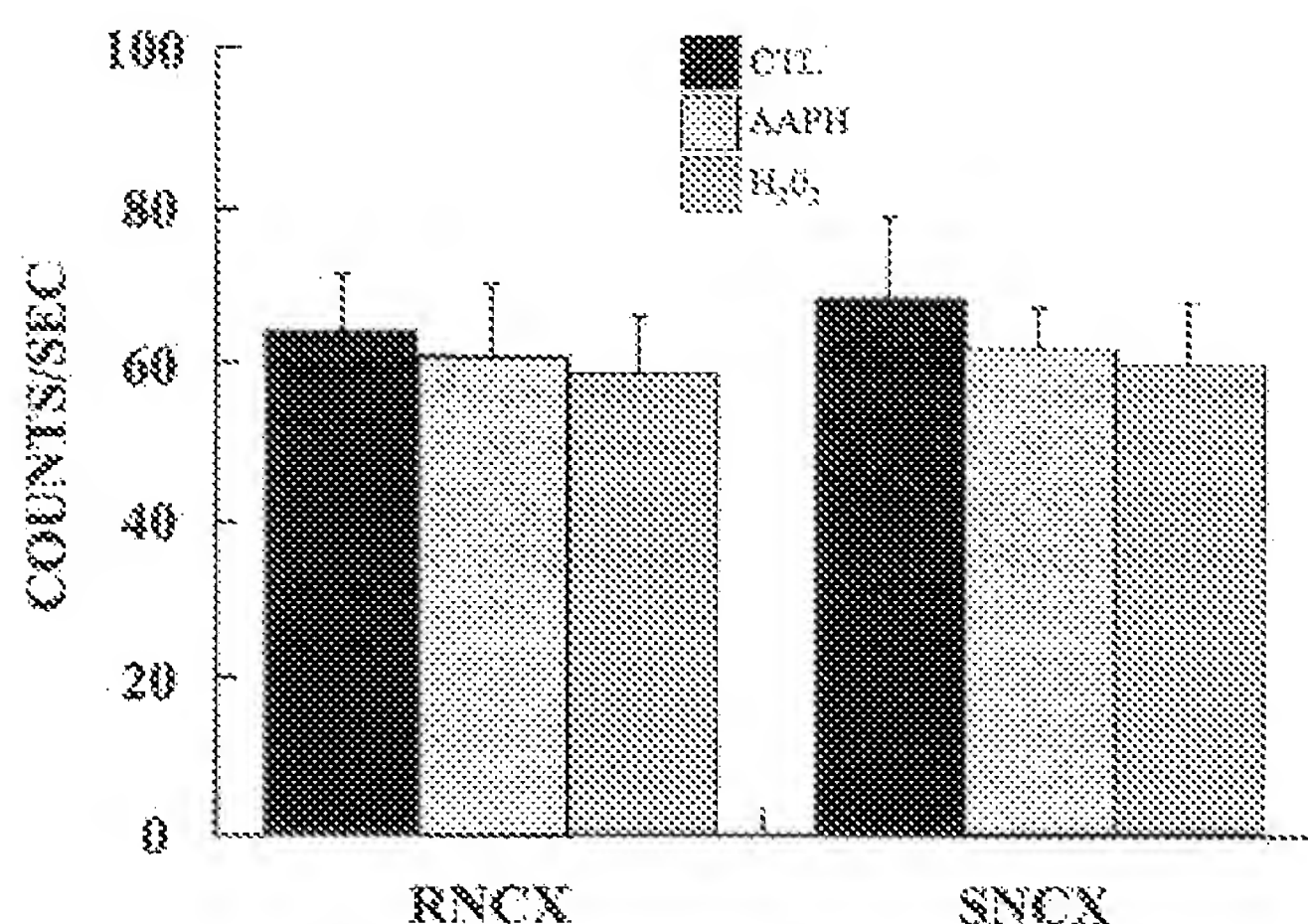


Figure 5. AAPH and H_2O_2 did not significantly affect cell viability. OK-PTH cells expressing RNCX or SNCX were plated on coverslips and grown to confluence. Coverslips were either left untreated or treated with AAPH (5 mg/mL) or H_2O_2 (750 $\mu\text{mol/L}$) for 30 minutes and perfused with Ringer's solution in the presence of the fluorescence dye BCECF. Ability of the cells to take up BCECF was monitored using dual-excitation-wavelength fluorescence spectroscopy (Photon Technologies International). Data are mean \pm SEM; $n=3$.

blockade of angiotensin II type I receptors attenuated oxidative stress significantly and tended to normalize the mean arterial pressure in these rats.¹⁵ In the salt-sensitive Dahl/Rapp rat, a high-salt (8% NaCl) diet resulted in elevated systolic pressure^{16,17} and oxidative stress^{16,18} and induced heart failure.¹⁶ The use of the angiotensin-converting enzyme inhibitor cilazapril, in the presence of a high-salt diet, resulted in lower blood pressure and oxidative stress and prevented heart failure.¹⁶

The exact mechanism(s) through which oxidative stress contributes to the etiology of hypertension is not fully understood. However, studies have shown that increased oxidative stress affects hypertension, in part by reducing the levels of nitric oxide (NO), which is generated through the metabolism of L-arginine.^{15,17,18} NO is an antihypertensive factor that stimulates dilation and relaxation of vascular smooth muscle cells, decreases platelet aggregation and adhesion, and decreases the growth of vascular smooth muscle cells.^{19,20} Thus, reduction in NO can lead to augmented vasoconstrictive responses, increased blood viscosity and resistance to blood flow, and hypertension.

Under oxidative stress conditions, NO depletion occurs by the reaction of NO with the superoxide anion (O_2^-) to form peroxynitrite (ONOO^-). The presence of peroxynitrite can lead to a number of adverse effects, including protein nitration,²¹ lipid peroxidation,²¹ DNA degradation,²¹ enhanced tubuloglomerular feedback responses,^{15,22} and dysregulation of $[\text{Ca}^{2+}]_i$ homeostasis.²³⁻²⁵ Elevation of $[\text{Ca}^{2+}]_i$ under oxidative stress conditions has been shown in mammalian cells, including rat fasciculate reticularis and glomerulosa cells,²⁶ distal colon and colon (CaCo) cells,^{27,28} leukemic (AML) cells,²⁹ pheochromocytoma (PC12) cells,²⁹ alveolar macrophages,³⁰ vascular endothelial cells,³¹ and vascular smooth muscle cells.³² In all of these cell types, induction of oxidative stress by a variety of conditions led to abnormal increases in $[\text{Ca}^{2+}]_i$. A number of studies have found that the adverse effects of oxidative stress can be attenuated by treatment with either calcium chelators, such as BAPTA [bis-(*o*-aminophenoxy)-ethane-*N,N,N',N'*-tetraacetic acid/tetraacetoxymethyl) or EGTA, the addition of antioxidative enzymes (superoxide dismutase and catalase), or treatment with antioxidants (vitamin E and uric acid). The attenuation of the adverse effects of oxidative stress through the use of calcium chelators is strong evidence that at least part of the deleterious effects of oxidative stress is mediated through elevations of $[\text{Ca}^{2+}]_i$. Therefore, current evidence suggests that dysregulation of $[\text{Ca}^{2+}]_i$, which has been attributed at least in part to a decrease in exchange of Ca^{2+} for Na^+ ,³³ might be 1 link between oxidative stress and hypertension.

The role of the renal NCX in the regulation of renal hemodynamics has been elegantly demonstrated in a recent study that showed that inhibition of afferent arteriole $\text{Na}^+/\text{Ca}^{2+}$ exchange increased renal vascular resistance.³⁴ Previous work from our laboratory has identified the presence of functional activity of the NCX in renal contractile cells.^{2,4} Through reverse transcription-polymerase chain reaction-based cloning, we identified functional and amino acid differences between renal contractile cell exchanger isoforms from SNCX and RNCX rats. Thus, we sought to examine the

effect that oxidative stress might have on the exchange activities of these 2 NCX isoforms. SNCX and RNCX show differences at the amino acid level, at the *N*-terminus at amino acid 218 ($I_{\text{RNCX}}/F_{\text{SNCX}}$), and at the alternative splice site, where this region is encoded by exons B and D in RNCX and by B, D, and F in SNCX. Our functional studies indicated that SNCX had an impaired ability to regulate agonist-induced $[\text{Ca}^{2+}]_i$ increases compared with RNCX. Therefore, the rationale of the present studies was as follows. When placed on a high-salt diet, the SNCX rat has been shown to generate excess reactive oxygen species and exhibits high levels of oxidative stress. There is also ample evidence for the dysregulation of $[\text{Ca}^{2+}]_i$ in this model of hypertension. Because the salt-sensitive rat expresses an altered form of the NCX with a compromised ability to regulate $[\text{Ca}^{2+}]_i$, dysregulation of $[\text{Ca}^{2+}]_i$ under oxidative stress conditions might be due, at least in part, to enhanced susceptibility of the SNCX isoform to oxidative stress. This enhanced susceptibility to oxidative stress would then reduce the ability of this exchanger isoform to regulate $[\text{Ca}^{2+}]_i$.

Using OK-PTH cells stably expressing either RNCX or SNCX, we showed by $^{45}\text{Ca}^{2+}$ -influx assays that assessed reverse-mode Na⁺/Ca²⁺ exchange (Ca^{2+} influx/Na⁺ efflux) that SNCX demonstrated an enhanced sensitivity to H₂O₂ and peroxynitrite compared with RNCX. Because the reverse mode is opposite to how the NCX normally operates under physiologic conditions, we next examined the effects of H₂O₂ and peroxynitrite on forward-mode Na⁺/Ca²⁺ exchange (Ca^{2+} efflux/Na⁺ influx) in cells expressing either SNCX or RNCX. This was accomplished by ratiometric fura-2 measurements to monitor changes in $[\text{Ca}^{2+}]_i$ in cells expressing either RNCX or SNCX in the presence or absence of H₂O₂ or peroxynitrite. Because the 2 cell lines are similar in every respect except in the NCX isoforms that they express, changes in basal $[\text{Ca}^{2+}]_i$ in the presence of H₂O₂ or peroxynitrite would reflect the abilities of these isoforms to regulate $[\text{Ca}^{2+}]_i$ in response to oxidative stress. In the presence of H₂O₂ or peroxynitrite, cells expressing SNCX showed significant increases in $[\text{Ca}^{2+}]_i$ compared with cells expressing RNCX. In addition, the initial rate at which $[\text{Ca}^{2+}]_i$ increased was ≈ 3 -fold greater in cells expressing SNCX compared with cells expressing RNCX. Thus, the significantly greater increase in $[\text{Ca}^{2+}]_i$ with oxidative stress in cells expressing SNCX reflects the impaired ability of this isoform to regulate $[\text{Ca}^{2+}]_i$. These studies are consistent with our recent work that demonstrated (1) regulation of basal, ie, nonstimulated, $[\text{Ca}^{2+}]_i$ by the NCX; specifically, expression of either RNCX or SNCX lowered baseline $[\text{Ca}^{2+}]_i$ in OK-PTH cells compared with nontransfected cells and (2) cells expressing SNCX had a reduced ability to regulate agonist (ATP)-induced increases in $[\text{Ca}^{2+}]_i$. In this regard, oxidative stress appears to be another example of an “agonist” or stimulus that has greater effects on $[\text{Ca}^{2+}]_i$ dynamics in cells expressing SNCX compared with cells expressing RNCX. Our results are also in agreement with previous studies that demonstrated that brief exposure of Chinese hamster ovary cells (CHO-1) expressing NCX1 to the peroxyl-radical-generating azo initiator AAPH and to peroxynitrite inhibited exchanger activity.²⁶ Inhibition of exchanger activity occurred as a result of aggregation of the

exchanger into high-molecular-weight complexes and exchanger fragmentation, which occurred under oxidative stress conditions.²⁶

To assess a possible mechanism through which oxidative stress attenuates exchanger activity, cells expressing RNCX or SNCX were treated with 750 $\mu\text{mol/L}$ H₂O₂ or 5 mg/mL AAPH for 30 minutes. AAPH was used instead of peroxynitrite because peroxynitrite was only stable for a few seconds. Total protein was isolated and immunoblotting was carried out with a rabbit polyclonal NCX antibody. Our results showed that high levels of oxidative stress attenuated the levels of both RNCX and SNCX proteins, although we were unable to determine whether this attenuation occurred as a result of protein fragmentation or aggregation. Therefore, our studies demonstrate that inhibition of exchanger activity under oxidative stress conditions occurs, at least in part, because of attenuation of NCX levels. Whether this results from protein degradation or aggregation requires further investigation.

Perspectives

Oxidative stress and dysregulation of $[\text{Ca}^{2+}]_i$ dynamics occur in hypertension. In the salt-sensitive Dahl/Rapp rat, a model of salt-sensitive hypertension, a high-salt diet resulted in elevated systolic pressure^{16,17} and oxidative stress^{16,18} and induced heart failure.¹⁶ An NCX isoform that is expressed in renal contractile cells of this rat (SNCX), which differs from an exchanger isoform that is expressed in renal contractile cells of the salt-resistant Dahl/Rapp rat (RNCX), showed enhanced sensitivity to oxidative stress. The enhanced sensitivity of this NCX isoform to oxidative stress might provide a basis for the hypertension and renal failure that are hallmarks of this model of salt-sensitive hypertension.

Acknowledgments

This work was supported by grants R01HL-50163 (to P.D.B.) and 1K01HL67718-01 (to M.T.U.) from the National Heart, Lung, and Blood Institute. The authors would like to thank Martha Yeager and Amanda Fuson for their assistance in preparing this manuscript.

References

1. Bell PD, Mashburn N, Unlap MT. Renal sodium/calcium exchange: a vasodilator that is defective in salt-sensitive hypertension. *Acta Physiol Scand*. 2000;168:209–214.
2. Mashburn NA, Unlap MT, Runquist J, Alderman A, Johnson GV, Bell PD. Altered protein kinase C activation of Na⁺/Ca²⁺ exchange in mesangial cells from salt-sensitive rats. *Am J Physiol*. 1999;276:F574–F580.
3. Nelson LD, Mashburn NA, Bell PD. Altered sodium-calcium exchange in afferent arterioles of the spontaneously hypertensive rat. *Kidney Int*. 1996;50:1889–1896.
4. Nelson LD, Unlap MT, Lewis JL, Bell PD. Renal arteriolar Na⁺/Ca²⁺ exchange in salt-sensitive hypertension. *Am J Physiol*. 1999;276:F567–F573.
5. Unlap MT, Peti-Peterdi J, Bell PD. Cloning of mesangial cell Na⁺/Ca²⁺ exchangers from Dahl/Rapp salt-sensitive/resistant rats. *Am J Physiol*. 2000;279:F177–F184.
6. Hwang EF, Williams I, Kovacs G, Peti-Peterdi J, Siroky B, Rice WC, Bates E, Schwiebert EM, Unlap MT, Bell PD. Impaired ability of the Na⁺/Ca²⁺ exchanger from the Dahl/Rapp salt sensitive rat to regulate cytosolic calcium. *Am J Physiol*. 2003;284:F1023–F1031.
7. Wu A, Derrico CA, Hatem L, Colvin RA. Alzheimer's amyloid- β peptide inhibits sodium/calcium exchange measured in rat and human brain plasma membrane vesicles. *Neuroscience*. 1997;80:675–684.

8. Wells IC, Blotcky AJ. Coexisting independent sodium-sensitive and sodium-insensitive mechanisms of genetic hypertension in spontaneously hypertensive rats (SHR). *Can J Physiol Pharmacol*. 2001;79:779–784.
9. Fenster CP, Weinsier RL, Darley-USmar VM, Patel RP. Obesity, aerobic exercise, and vascular disease: the role of oxidant stress. *Obes Res*. 2002;10:964–968.
10. Franco Mdo C, Dantas AP, Akamine EH, Kawamoto EM, Fortes ZB, Scavone C, Tostes RC, Carvalho MH, Nigro D. Enhanced oxidative stress as a potential mechanism underlying the programming of hypertension in utero. *J Cardiovasc Pharmacol*. 2002;40:501–509.
11. Huschenbett J, Zaidi A, Michaelis ML. Sensitivity of the synaptic membrane $\text{Na}^+/\text{Ca}^{2+}$ exchanger and the expressed NCX1 isoform to reactive oxygen species. *BBA Biomembranes*. 1998;1374:34–46.
12. Kaplan P, Matejovicova M, Mezesova V. Iron-induced inhibition of Na^+/K^+ -ATPase and $\text{Na}^+/\text{Ca}^{2+}$ exchanger in synaptosomes: protection by the pyridindole stobadine. *Neurochem Res*. 1997;22:1523–1529.
13. Grynkiewicz G, Poenie M, Tsien RY. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J Biol Chem*. 1985;260:3440–3450.
14. Sellers JR, Cook S, Goldmacher VS. A cytotoxicity assay utilizing a fluorescent dye that determines accurate surviving fractions of cells. *J Immunol Methods*. 1994;172:255–264.
15. Welch WJ, Wilcox CS. AT1 receptor antagonist combats oxidative stress and restores nitric oxide signaling in the SHR. *Kidney Int*. 2001;59:1257–1263.
16. Tsutsui H, Ide T, Hayashidani S, Kinugawa S, Suematsu N, Utsumi H, Takeshita A. Effects of ACE inhibition on left ventricular failure and oxidative stress in Dahl salt-sensitive rats. *J Cardiovasc Pharmacol*. 2001;37:725–733.
17. Chen PY, St John PL, Kirk KA, Abrahamson DR, Sanders PW. Hypertensive nephrosclerosis in Dahl/Rapp rat. *Lab Invest*. 1993;68:174–184.
18. Trolliet MR, Rudd MA, Loscalzo J. Oxidative stress and renal dysfunction in salt-sensitive hypertension. *Kidney Blood Press Res*. 2001;24:116–123.
19. Serrano Rios M. Relationship between obesity and the increased risk of major complications in non-insulin-dependent diabetes mellitus. *Eur J Clin Invest*. 1998;28:14–18.
20. Britten MB, Zeiher AM, Schachinger V. Clinical importance of coronary endothelial vasodilator dysfunction and therapeutic options. *J Intern Med*. 1999;245:315–327.
21. Hemnani T, Parihar MS. Reactive oxygen species and oxidative DNA damage. *Indian J Physiol Pharmacol*. 1998;42:440–452.
22. Welch WJ, Wilcox CS. Role of nitric oxide in tubuloglomerular feedback: effects of dietary salt. *Clin Exp Pharmacol*. 1997;24:582–586.
23. Kmonickova E, Kamenikova L, Hynie S, Farghali H. Cyclosporin A modifies cytoplasmic calcium levels in isolated hepatocytes exposed to oxidative stress due to *tert*-butyl hydroperoxide. *Physiol Res*. 2000;49:471–474.
24. Han B, Klonowski-Stumpe H, Luthen R, Schreiber R, Haussinger D, Niederau C. Menadione-induced oxidative stress inhibits cholecystokinin-stimulated secretion of pancreatic acini by cell dehydration. *Pancreas*. 2000;21:191–202.
25. Dreher D, Junod AF. Differential effects of superoxide, hydrogen peroxide, and hydroxyl radical on intracellular calcium in human endothelial cells. *J Cell Physiol*. 1995;162:147–153.
26. Garcia R, Enriquez de Salamanca A, Portoles MT. Calcium and reactive oxygen species as messengers in endotoxin action on adrenocortical cells. *BBA Mol Basis Dis*. 1999;1454:1–10.
27. Karczewski JM, Peters JG, Noordhoek J. Prevention of oxidant-induced cell death in Caco-2 colon carcinoma cells after inhibition of poly(ADP-ribose) polymerase and Ca^{2+} chelation: involvement of a common mechanism. *Biochem Pharmacol*. 1999;57:19–26.
28. Abrahamse SL, Pool-Zobel BL, Rechkemmer G. Potential of short chain fatty acids to modulate the induction of DNA damage and changes in the intracellular calcium concentration by oxidative stress in isolated rat distal colon cells. *Carcinogenesis*. 1999;20:629–634.
29. Hedley DW, McCulloch EA, Minden MD, Chow S, Curtis J. Antileukemic action of buthionine sulfoximine: evidence for an intrinsic death mechanism based on oxidative stress. *Leukemia*. 1998;12:1545–1552.
30. Hoyal CR, Giron-Calle J, Forman HJ. The alveolar macrophage as a model of calcium signaling in oxidative stress. *J Toxicol Environ Health B*. 1998;1:117–134.
31. Panayiotidis M, Tsolas O, Galaris D. Glucose oxidase-produced H_2O_2 induces Ca^{2+} -dependent DNA damage in human peripheral blood lymphocytes. *Free Radic Biol Med*. 1999;26:548–556.
32. Ishizaka N, Griendling KK. Heme oxygenase-1 is regulated by angiotensin II in rat vascular smooth muscle cells. *Hypertension*. 1997;29:790–795.
33. Dhalla NS, Golfman L, Takeda S, Takeda N, Nagano M. Evidence for the role of oxidative stress in acute ischemic heart disease: a brief review. *Can J Cardiol*. 1999;15:587–593.
34. Schweda F, Seebauer H, Kramer BK, Kurtz A. Functional role of sodium-calcium exchange in the regulation of renal vascular resistance. *Am J Physiol*. 2001;280:F155–F161.